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# Human Herpesvirus 6 Induces IL-8 Gene Expression in Human Hepatoma Cell Line, Hep G2

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The infectivity of human herpesvirus 6 (HHV-6) in a human hepatoma cell line, Hep G2 cells, and the effect of HHV-6 on production of inflammatory cytokines in these cells were examined to analyze pathogenesis of HHV-6 in the liver. We demonstrated that Hep G2 cells were susceptible to infection with HHV-6, and produced infectious virus. Moreover, infection of Hep G2 cells by HHV-6 induced the expression of IL-8 mRNA, but not IL-1β. The effect on induction of IL-8 gene expression was observed only in Hep G2 cells infected with infectious virus, whereas both heat-inactivated HHV-6 and UV-irradiated HHV-6 did not change the IL-8 mRNA level in these cells. These data suggest that HHV-6 may induce the cytokine-mediated inflammatory response by infecting liver cells, which could result in liver dysfunction in vivo. 

**KEY WORDS:** human herpesvirus 6 (HHV-6), hepatocytes, IL-8, IL-1β cytokine-mediated inflammation, liver dysfunction

## INTRODUCTION

Human herpesvirus 6 (HHV-6) has been isolated by several groups from patients with lymphoproliferative disorders or AIDS [Salahuddin et al., 1986; Tedder et al., 1987; Downing et al., 1987; Agut et al., 1988; Lopez et al., 1988]. Until now, the distinct nature of HHV-6 as compared with other herpesviruses was confirmed by molecular and immunological analyses [Josephs et al., 1986]. HHV-6, which is genetically classified into two variants (HHV-6A and HHV-6B) [Ablashi et al., 1993], is also ubiquitous like other herpesviruses, and primary infection is observed from 6 months to 2 years [Briggs et al., 1988; Saxinger et al., 1988; Okuno et al., 1989]. This virus infects and replicates mainly in cells of the immune system, e.g., namely $CD4^+$ T cells, monocytes/macrophages, and B cells [Lusso et al., 1988; Takahashi et al., 1989; Levy et al., 1990; Kondo et al., 1991]. Moreover, it has also been reported that the monocytes/macrophages is one of the target cells for latent infection [Kondo et al., 1991]. This fact suggests that infection or reactivation of HHV-6 is a causative factor of immunological disturbance in vivo. Although the details of HHV-6 pathogenesis are still unclear, it was demonstrated that HHV-6B is a causative agent for exanthem subitum (ES), also known as roseola infantum [Takahashi et al., 1988; Yamanishi et al., 1988]. This is one of the acute infant diseases characterized by high fever for 2–4 days, and the appearance of a diffuse maculopapular eruption after the fever subsides. In some cases, liver dysfunction was observed in the febrile phase of ES patients [Asano et al., 1990; Tajiri et al., 1990], and it was the symptom of liver dysfunction during the febrile phase that prompted us to investigate how HHV-6 might affect liver function. As a result of the fact that HHV-6 induces inflammatory cytokines in peripheral blood mononuclear cells [Kikuta et al., 1990; Flamand et al., 1991, 1995], and that liver cells are one of the "nonimmune" cells secreting these cytokines [Thornton et al., 1990; Tovey et al., 1991], we hypothesize that: (1) HHV-6 may infect liver cells directly, (2) act as an inflammatory cytokine inducer in these cells, and (3) lead to cytokine-mediated inflammation. To analyze this possibility, we investigated the infectivity of HHV-6 in a liver cell line, Hep G2 cells, and the effect of HHV-6 on cytokine syntheses by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. We concluded that Hep G2 cells were susceptible to HHV-6 infection, which induced IL-8 mRNA syntheses, but did not induce IL-1β, following an infection.

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## MATERIALS AND METHODS

### Virus Preparation

The HST strain of HHV-6B used for this study was isolated from a patient with ES [Takahashi et al., 1988]. To prepare the virus solution, HHV-6 was propagated in umbilical cord blood mononuclear cells (CBMC) stimulated with phytohemagglutinin (PHA) (5 μg/ml, Honen Oil Co.) for 2 days. First, CBMC ($1–3 \times 10^7$) were suspended with a virus solution having a titer of $5 \times 10^6$ 50% tissue culture infectious dose ($TCID_{50}$), centrifuged at 800 g for 40 min for adsorption, and cultured at 37°C in a $CO_2$ incubator with RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS). When >80% of CBMC infected with HHV-6 were antigen-positive by indirect immunofluorescence antibody assay (IFA) with mouse monoclonal antibodies to HHV-6 (see IFA section in Materials and Methods for details), the cell suspension was quickly frozen and thawed, and obtained cell lysate was used as the virus solution. CBMC lysate which was not infected with HHV-6 was also prepared and used instead of the virus solution as a mock infection. The virus solution and CBMC lysate used in this study were found to contain <10 pg/ml of endotoxin by a kit (Toxicolor® system: Seikagaku Corp., Tokyo, Japan).

### Inoculation of Hep G2 Cells With HHV-6

Hep G2 cells ($1 \times 10^6$), a human hepatoma cell line, were incubated with $1 \times 10^7$ $TCID_{50}$ of HHV-6, UV-irradiated HHV-6 (200 mW/cm², 4 min), heat-inactivated HHV-6 (56°C, 1 hr) or CBMC lysate instead of HHV-6 at 37°C for 1 hr. After the virus solution was discarded, the cells were cultured with RPMI 1640 medium containing 2% FCS at 37°C in a $CO_2$ incubator and harvested by treatment with trypsin at the various times after inoculation. Culture medium was changed at 1 day after inoculation to reduce the extracellular virus.

### Indirect Immunofluorescence Antibody Assay (IFA)

To detect HHV-6-infected Hep G2 cells, IFA was performed according to the previous method using mouse monoclonal antibodies against HHV-6 (OHV-1, -2 and -3) [Okuno et al., 1990, 1992].

### Assay for $TCID_{50}$

After inoculation of Hep G2 cells with HHV-6 as described above, the cells were collected at the various times after inoculation, and resuspended with 1 ml of RPMI 1640 medium. These were frozen at −80°C, and thawed immediately at 37°C. The cell lysates obtained were diluted with RPMI 1640 medium containing 10% FCS, and subsequently incubated with PHA-stimulated CBMC ($1 \times 10^6$) at 37°C in a $CO_2$ incubator for 7 days. Quadruplicate experiments were performed with each dilution. Virus antigens in each dilution were then checked by the IFA method, and the 50% endpoint was calculated by the method of Reed and Muench [1938].

### Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

The expression of interleukin (IL)-1β and IL-8 mRNA was analyzed by RT-PCR. Total RNA was isolated and precipitated from Hep G2 cells ($5 \times 10^5$), inoculated with or without HHV-6, by a single-step guanidinium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987], and 20% of the RNA was reverse-transcribed using random primers (Takara, Kyoto, Japan) with 200 units of RNase H-free reverse transcriptase (Superscript; GIBCO BRL, Gaithersburg, MD) as described previously [Miyata et al., 1994]. PCR amplification was performed in a total volume of 50 μl (with 2 μl of the reverse-transcribed products) containing 10 mM Tris hydrochloride (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM each of the four deoxynucleoside triphosphates, 0.001% gelatin, 100 pmol sense and antisense primers and 2.5 units AmpliTaq™ DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). The oligonucleotides primer sequences are as follows: Primers for IL-1β were 5′-ATGGCAGAAGTACCTGAGCTC-3′ and 5′-TTCCTTGAGGCCCAAGGCCAC-3′. The full-length amplified fragment is 540 bp long. IL-8 primers which amplified a 249 bp fragment were 5′-TCTGCAGCTCTGTGTGAAGG-3′ and 5′-ATTCTCAGCCCTCTTCAAAA-3′. The primers for elongation factor (EF) were 5′-CAAAGAATCTAGATGCAATAA-3′ and 5′-TGCCCATTAACAACAACAATCTG-3′ and amplified a 250 bp fragment. The EF served as an internal RNA control to allow comparison of RNA levels among different specimens. Specimens were amplified in a DNA Thermal cycler (Perkin Elmer Cetus, Norwalk, CT) for either 27 cycles (for mRNA of cytokines) or 20 cycles (for EF mRNA) consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. In preliminary experiments, reverse transcription and PCR amplification were performed on RNA from $10^4$, $10^5$, $10^6$ and $10^7$ cells of Hep G2 for 18, 21, 25, 28, 31, and 34 cycles. These experiments showed that with 27 cycles of amplification for cytokine mRNA, and with 20 cycles of EF mRNA amplification, the differences in PCR product signal were quantitatively related to input RNA. An aliquot of PCR products was resolved by electrophoresis in a 2% Seakem™ GTG agarose gel 0.4-cm-thick (FMC BioProducts, Rockland, ME), stained with ethidium bromide (0.5 μg/ml) for 30 min, and then destained with distilled water for 1 hr. PCR products were quantified precisely by measuring the signal intensity with a quantitation program for a fluorescent-bioimage analyzer (FMBIO, Takara Shuzo Co., Ltd., Kyoto, Japan). The intensity of individual bands was expressed as a relative area.

## RESULTS

### HHV-6-Infection of Hep G2 Cells

Hep G2 cells ($1 \times 10^6$), inoculated with or without $1 \times 10^7$ $TCID_{50}$ of HHV-6, were collected by treatment with trypsin at various days after inoculation and then examined for the presence of virus antigen by IFA. When Hep G2 cells were inoculated with virus, antigen-positive cells were first detected at 3 days postinfection. The

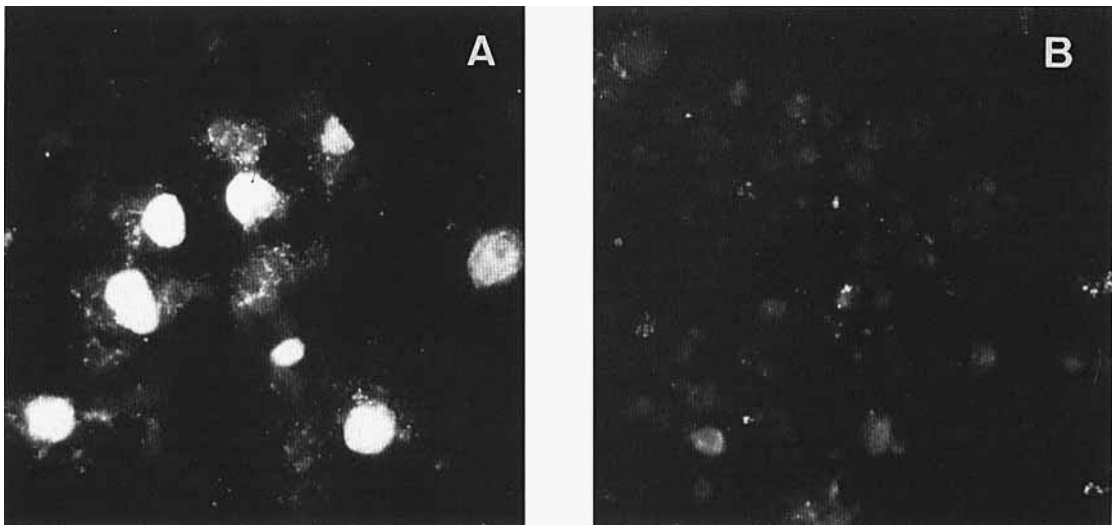


Fig. 1. HHV-6 infection of Hep G2 cells. Hep G2 cells ($1 \times 10^6$) were inoculated with or without $1 \times 10^7$ $TCID_{50}$ of HHV-6 (HST strain). The cells were harvested by trypsin treatment and examined for viral antigen by IFA using mouse monoclonal antibodies to HHV-6 at 5 days postinfection. **A:** Hep G2 cells inoculated with HHV-6. **B:** Mock-infected Hep G2 cells. Magnification, ×400.

number of antigen-positive cells gradually increased, and from the results of more than five separate experiments, approximately 20% of HHV-6-infected cells were detected at 5 days postinfection. At this time, although virus antigens were seen in the cytoplasm and nucleus (Fig. 1A), no specific antigen was detected in mock-infected culture (Fig. 1B). The staining pattern was similar to that observed with CBMC inoculated with HHV-6. Microscopic analysis of Hep G2 cell inoculation with HHV-6 did not show a cytopathic effect at any time. The similar results were observed when we used low passaged HHV-6 (<5 passages), or another human hepatoma cell line, Hep 3B (data not shown).

### HHV-6 Replication in Hep G2 Cells

In order to test whether or not Hep G2 cells produce the infectious virus after HHV-6 infection, cell lysates of Hep G2 cells inoculated, with or without HHV-6, at the various times after inoculation were prepared, and the amount of infectious virus in each cell lysate measured by $TCID_{50}$ assay. The results in Figure 2 show that Hep G2 cells produce HHV-6 until 5 days after infection, after which time the level of viral production declined gradually. This phenomenon demonstrates that Hep G2 is capable of producing infectious virus.

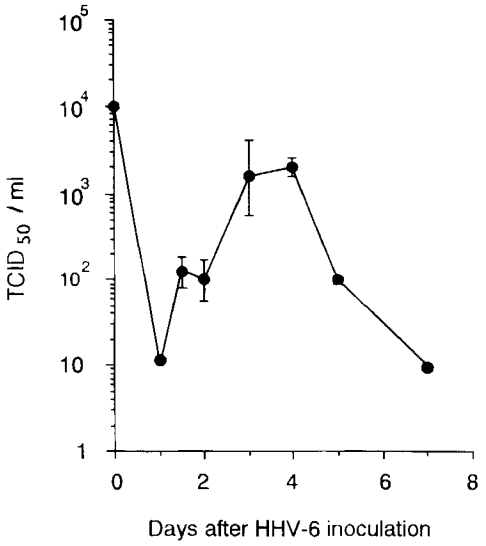


Fig. 2. HHV-6 replication in Hep G2 cells. Cell lysates obtained from HHV-6- or mock-infected Hep G2 cells ($1 \times 10^6$) at various points after inoculation were prepared, and the amount of infectious virus in each cell lysate was measured by $TCID_{50}$ assay. The values are expressed as means + range of the quadruplicate determinations. See Materials and Methods for details.

### Increase in the Level of IL-8, But Not IL-1β mRNA in Hep G2 Cells Inoculated With HHV-6

We examined the level of mRNA of inflammatory cytokines (IL-1β and IL-8) in Hep G2 cells, inoculated with or without HHV-6 at the various days after infection, by semiquantitative RT-PCR analysis. As shown in Figure 3, mRNA expressions of IL-8, but not IL-1β, were markedly increased in Hep G2 cells inoculated with HHV-6. Conversely, in the case of mock infection, the mRNA levels of each cytokine in Hep G2 cells by HHV-6 inoculation did not change significantly at any time (Fig. 3). Samples of RNA that had not been reverse-transcribed did not yield the PCR product, confirming the absence of the extraneous cDNA or PCR product contaminating the samples (data not shown). Peak levels of IL-8 mRNA expression were observed at 6–7 days after infection (Fig. 3C). The kinetics of IL-8 mRNA level is relatively coincident with that of virus replication

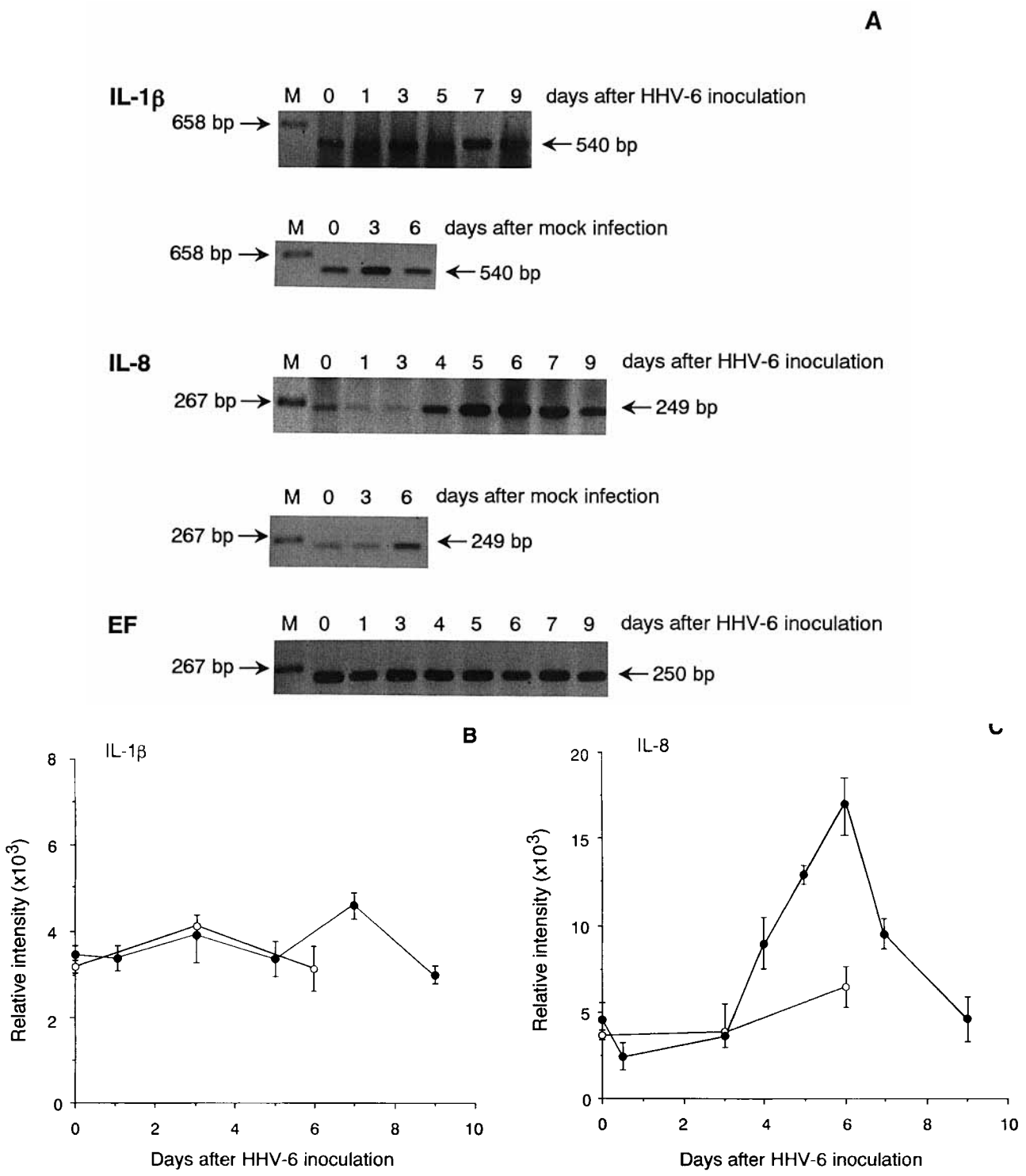


Fig. 3. Effect of HHV-6 infection on mRNA syntheses of inflammatory cytokines (IL-1β, and IL-8) by Hep G2 cells. **A:** Total RNA from Hep G2 cells inoculated with HHV-6 or CBMC lysate (mock) was harvested at various days postinfection and was reverse-transcribed. Then, IL-1β, IL-8 and EF cDNAs were amplified by PCR, analyzed on a 2% agarose gel, and visualized by ethidium bromide staining. The signal was detected by a fluorescent-bioimage analyzer, FMBIO. Representative data from the three separate experiments are shown. M, Marker fragments (pHY Marker). See Materials and Methods for details. **B:** Kinetics of IL-1β mRNA expression by Hep G2 cells following HHV-6 inoculation (filled circles) or mock infection (open circles). Each IL-1β PCR product was quantified precisely by measuring the signal intensity with a quantitation program for FMBIO, and then standardized against EF mRNA. The intensity of individual bands was expressed as relative area, and the levels are expressed as mean ± range of the triplicate determinations. See Materials and Methods for details. **C:** Kinetics of IL-8 mRNA expression by Hep G2 cells following HHV-6 inoculation (filled circles) or mock infection (open circles). Each IL-8 PCR product was quantitated as described above.

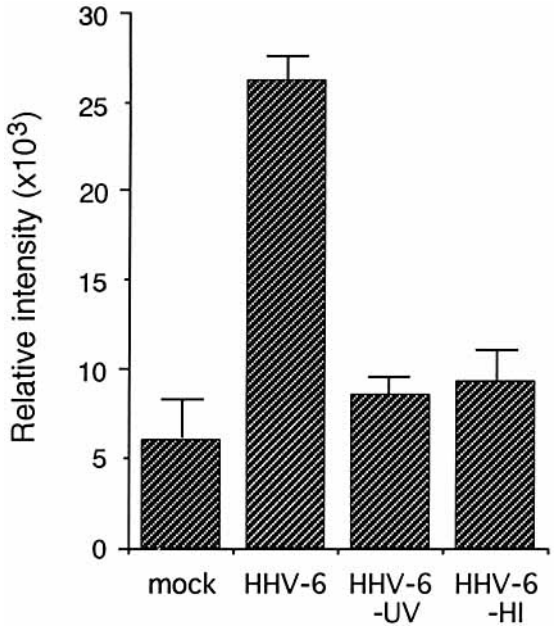


Fig. 4. Hep G2 cells ($1 \times 10^6$) were inoculated with $1 \times 10^7$ $TCID_{50}$ of infectious HHV-6, UV-irradiated HHV-6 (HHV-6-UV), heat-inactivated HHV-6 (HHV-6-HI) or CBMC lysate (mock) and then collected by trypsin treatment at 5 days postinfection. Total RNA from each cells was reverse-transcribed, and IL-8 and EF cDNAs were amplified by PCR. Then, each PCR product was quantitated as described in the legend to Figure 3B. The values are expressed as means ± range of the triplicate determinations. See Materials and Methods for details.

in Hep G2 cells (Fig. 2), suggesting that the change of IL-8 mRNA level seems to be associated with viral replication. Consequently, these results demonstrate that HHV-6 inoculation increases the level of IL-8 gene expression in Hep G2 cells.

### Inoculation With Heat-Inactivated HHV-6 or UV-Irradiated HHV-6 did not Upregulate mRNA Expression of IL-8 in Hep G2 Cells

Next, to analyze whether or not the induction of IL-8 gene expression in Hep G2 cells inoculated with HHV-6 requires viral replication, we examined the ability of heat-inactivated or UV-irradiated HHV-6 to affect the expression of IL-8 mRNA in Hep G2 cells. While infectious HHV-6 induced IL-8 mRNA expression at 5 days postinfection as before (Fig. 3C), neither heat-inactivated HHV-6 nor UV-irradiated HHV-6 showed this effect on cytokine induction (Fig. 4). Furthermore, the level of IL-8 mRNA did not change at any time following inoculation with either heat-inactivated HHV-6 or UV-irradiated HHV-6 (data not shown).

## DISCUSSION

Although ES caused by primary HHV-6 infection is normally an acute, but mild infant disease [Takahashi et al., 1988; Yamanishi et al., 1988], liver dysfunction also has been observed following an infection [Asano et al., 1990; Tajiri et al., 1990]. To analyze pathogenesis of HHV-6 in the liver, we examined the infectivity of HHV-6 in liver cells, and the effect of HHV-6 on production of inflammatory cytokines in these cells. The results in this paper showed that: (1) the human hepatoma cell line, Hep G2, was susceptible to infection with HHV-6, and produced the infectious virus; (2) inoculation of Hep G2 cells by HHV-6 induced mRNA expression of the inflammatory cytokine, IL-8, but not IL-1β; and (3) the induction of IL-8 gene expression was observed only in Hep G2 cells inoculated with infectious HHV-6, whereas neither heat-inactivated HHV-6 nor UV-irradiated HHV-6 had this effect. These findings support our hypothesis that HHV-6 infects liver cells directly, and subsequently, affects liver function.

Recently, some investigators have suggested that some inflammatory cytokines play an important role in liver dysfunction, and the mechanisms of the onset of viral hepatitis are strongly implicated in the abnormal immune response such as the change of some inflammatory cytokine syntheses [Tovey et al., 1991; Sun et al.,1992; Al-Wabel et al., 1993; Torre et al., 1994]. For example, IL-8, one of the most potent chemoattractants for neutrophils and leukocytes [Larsen et al., 1989; Strieter et al., 1989], is upregulated at the mRNA level in liver cirrhosis [Napoli et al,. 1994]. Hill et al. [1993] reported that plasma IL-8 concentration increased in patients with alcoholic hepatitis. In addition, hepatitis B virus X protein can transactivate the IL-8 gene by acting on nuclear factor κB (NF-κB) and CCAAT/enhancer-binding protein-like cis-elements, and might play a role in the pathogenesis of inflammation in viral hepatitis [Mahe et al., 1991]. These findings, together with our present observation, indicate the possibility that HHV-6 might act as an inducer of inflammatory cytokines such as IL-8, which could lead to liver dysfunction due to the cytokine-mediated inflammatory response. Moreover, Hasegawa et al. [1994] demonstrated that the infection of HHV-6 in T lymphocytes induces the expression of EBI 1, which is a putative G protein-coupled peptide receptor that shows >30% amino acid identity to IL-8 receptors. Although the ligand for EBI 1 is unknown, one possibility considered is that induction of EBI 1 may enhance the migration of HHB-6-infected T cells to liver secreting IL-8 by HHV-6 infection.

On the other hand, gene expression of IL-1β in Hep G2 cells did not change by HHV-6 infection at any time, in contrast with that of IL-8. Thornton et al. [1990] demonstrated that Hep G2 cells are capable of IL-8 production in response to IL-1β. Of particular interest in our results is the fact that the change of IL-8 gene expression in Hep G2 cells infected with HHV-6 did not correlate with that of IL-1β. Although the mechanism(s) of this phenomenon is unclear, certain viral factor(s) of HHV-6 may directly induce IL-8 gene expression without the regulation of IL-1β. It may seem like that the hepatitis B virus X protein directly induces IL-8 production [Mahe et al., 1991].

Flamand et al. [1991] previously reported that HHV-6 variant A (GS strain) induces IL-1β production in

peripheral blood mononuclear cells, and that viral replication is not required for this effect. These findings are different from our results, which demonstrated that HHV-6 variant B (HST strain) did not induce IL-1β mRNA expression in hepatocytes. A possibility of this discrepancy may be due to the difference in the HHV-6 variant examined as a result of the differences in the pathogenesis between HHV-6 variants A and B. In fact, it is well-known that primary infection of HHV-6 variant B causes ES [Takahashi et al., 1988; Yamanishi et al., 1988], whereas HHV-6 variant A has never been found to be the cause of ES. As yet, the pathogenesis of variant A is still unknown. Another possibility is that HHV-6 may affect cytokine synthesis by different mechanism(s) in hepatocytes and lymphocytes. Although Hep G2 cells required viral replication for upregulation of IL-8 gene expression, lymphocytes did not require viral replication for upregulation of IL-1β. Further investigations will be necessary to clarify this point, as little is known about the effect of HHV-6 on cytokine synthesis at the present time.

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